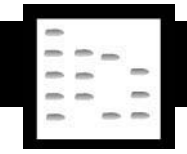
ENZYMES



Chromatography

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Separation and Determination in Physiological Samples

The determination of an absolute enzyme concentration in a physiological sample is principally straightforward; the main problem is the need for a purified sample to use as a standard.

Enzymes are proteins found in nature in complex mixtures, usually in cells which perhaps contain several hundreds of different enzymes. In order to understand and interpret enzyme data from complex biological systems in, for instance, a subcellular organelle (such as a mitochondrion), a cell or whole organism, we must try to understand its properties in as simple a system as possible. From studies of an isolated enzyme we can learn about its specificity for certain substrates, the kinetic parameters for the reaction and the possible means of regulation. All these parameters are useful for understanding the role of the enzyme in more complex systems. The ready availability of isolated enzymes has been of considerable value in a number of medical and industrial applications.

To study a given enzyme properly in physiological samples it must be purified. Maintenance of biological activity is the goal throughout the whole purification scheme. Extracellular enzymes usually withstand the variety of stresses they are exposed to during the purification. In contrast, when released from their natural protective environment, intracellular enzymes are very sensitive to various steps in the purification scheme. Integral membrane enzymes are especially vulnerable during solubilization.

Specific examples, together with trends in enzyme determination in physiological samples, will be discussed in this article.

Preparation of Enzymes with Biological Activity from Physiological Samples

Enzyme Activity Measurement Process

To elucidate enzymatic activity from physiological samples certain steps have to be performed.

1. Preparation of reaction mixture and enzyme, where the reaction mixture usually consists of a controlled substrate solution with the correct temperature, pH and any cofactors needed for catalysis. Enzymes often demand a more complex preparation methodology than substrates and the reaction mixture. This will be discussed later.
2. Initiation and incubation, which are usually started by adding the proper enzyme preparation to the reaction mixture or vice versa. All subsequent time measurements are related to this initial time.
3. Termination, which can be achieved in various ways. Normally, this means inactivation of the catalytic activity of the enzyme.
4. Separation of the enzyme products from the enzyme and its substrates.
5. Detection and indetification of the enzymatically formed product(s) during specific incubation intervals.
6. Under certain conditions enzyme activity can be followed dynamically, i.e. by rate measurement ($\Delta f/\Delta t$, where f is temperature, absorbance, fluorescence, etc., and t is time).
7. Interpretation of the produced data.

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Handling of Specimens and Samples (the Preanalytical Phase)

For all biological material (tissue, urine, cerebrospinal fluid, cell cultures, etc.) the same basic sequence of procedures applies.

1. Preparation of subject to be investigated.
2. Collection of specimen.
3. Separation of sample from specimen.
4. Transport of specimen and/or sample.
5. Storage of specimen and/or sample.
6. Pretreatment of samples for enzymatic analysis.

The specimen is defined as that part of the subject which is taken as representative for the analysis. The sample is the material that is actually analysed. The sample can be derived from, prepared from or be a part of the specimen which is homogenized for measurement of enzyme activities. Consequently the aliquot of homogenate that is analysed is the sample. Only under certain conditions is the sample identical to the specimen.

Sample Preparation Strategy

Two factors should be considered during preparation of enzymes with biological activity from physiological samples. The first factor for consideration is the selection of the biological sample that is to be used as the starting material for the purification. The samples can be subdivided into three groups, depending on their complexity (see **Figure 1**).

The first group (I) includes samples containing different cell types and extracellular compartments, e.g. samples containing organs, tissue, biological fluids, microbial cells, and unicellular organisms from a culture medium or fermentation broth. Initially cellular compartments have to be separated from noncellular compartments. The second group (II) consists of different cell types within the cellular compartments being separated form each other. Group II samples are thus homogeneous populations of each type of cell. This becomes the starting material for samples where cell-surface activities can be directly assayed or the cells can be lysed (broken up), thus providing access to the activities in intracellular organelles and on cytoplasmic fragments.

The third group (III) consists of subcellular fragments liberated by lysis of group II samples. These fragments include organelles such as mitochondria as well as those operationally defined as the membrane fraction or a fraction containing soluble components. The initial steps within this group will be separation of different organelles and separation of soluble from insoluble material. This is followed by solubilization

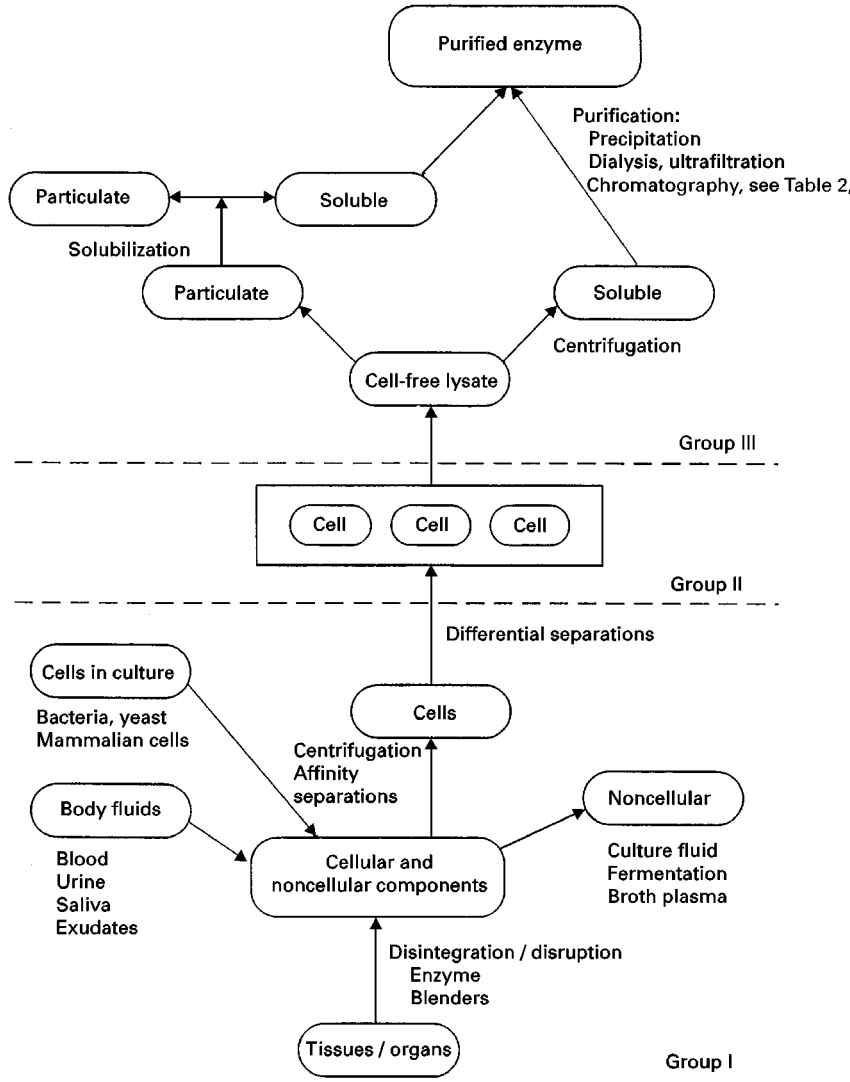

Figure 1 Enzyme purification scheme. Samples can be divided into three groups, I, II and III, depending on their complexity. Samples in the different groups enter and leave the purification process at different points. The samples in group I are the most complex, consisting of both extracellular and cellular enzyme-containing material which must first be separated. Group II samples contain different types of cells, one of which contains the enzyme of interest. In group III the enzyme source is from only one type of cell, from which the 'right' subcellular fraction gives the 'right' enzyme. (Adapted from Rossomando, 1987.)

of membrane fractions and finally separation of molecular species form each other.

The second factor for consideration during the preparation of biologically active enzymes concerns to what extent the sample should be purified. The traditional end point of any purification scheme would be a homogeneous protein. The main goal should be to assay a single enzymatic activity without interference form other activities. However, for some studies it is advantageous or even necessary to assay the activity of interest in the presence of other activities.

Sample Obtained from Tissue or Organ

Tissues and organs (e.g. skin, liver) can be divided into at least two compartments, the cellular compartment and the extracellular compartment. The activity of interest could be localized in either of the compartments. Cell-sorting techniques should be used where whole undamaged cells are separated from the stable fibrillar matrix. Some cell damage is unavoidable with the harsh methods often used for disrupting the matrix, e.g. cutting or dicing with scissors, shearing in a blender, or grinding. Specific disruption of the matrix can be performed using purified enzymes, e.g. collagenase for mammalian tissues. Trypsin and other proteolytic enzymes have also been used with success. Such procedures result in a mixture of cells extracellular compartments including some insoluble fragments, and added enzymes (including reagents).

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Samples Obtained from Tissue or Organ Culture

Cultured samples are usually treated in the manner described previously. Precautions must be taken to avoid errors due to the additional extracellular compartments and the culture medium, which may contain enzymatic activities originating from the medium itself or produced during sample growth.

Samples Obtained from Biological Fluids

Body fluids such as blood, cerebrospinal fluid and saliva contain cells as a normal component. However, in other fluids cells are a contamination. Cells in urine may indicate a disease process. The study of enzymes in such fluids also requires separation of the two compartments (cells from biological fluid). Because biological fluids do not contain fibrillar matrix material, the separation will, for instance, be a simple centrifugation step (5000 *g* for 15 min) which will produce a pellet containing most of the cellular material. The supernatant produced by centrifugation can be assayed for enzymatically active proteins.

Samples Obtained from Cell Culture

For cells grown in liquid culture, including mammalian cells, fungi, protozoa and bacteria, noncellular compounds should be separated form the cells before analysis takes place. Even here, a low-speed centrifugation is sufficient for separation of cells from culture media. The supernatant should be assayed for enzymatic activity and the cell-pellet material could be set aside for later assay or lysis.

Enzyme Activity Determination

Extracellular Enzymes

The extracellular fluid around tissues or the growth medium around mammalian cells, bacteria, yeast or fungi cells often contains the enzyme activity of interest. Several factors have to be considered before activity measurements can be started. Proteolytic enzymes must be inhibited early in the determination process, because otherwise they will degrade and destroy the enzymatic activity of interest. Another factor for consideration is the interference of small molecules, which could be erroneously measured as enzyme substrate or product. A special case is inhibitors which diminish enzyme activity. If a serum-free medium is not used during mammalian cell propagation, special care concerning serum enzymatic activity measurements and purification has to be taken.

Within the Cellular Compartment

After sample preparation, cells are separated from noncellular material in different manners depending on the complexity of the original sample (i.e. from which organ the sample originates). Because a number of different types of cells could remain, an assay of any complex sample should begin with the preparation of only one cell type.

Many separation methods utilizing different properties of the cells have been used. In sucrose gradient centrifugation, density differences between cell types are used and each cell type finds its equilibrium position in the sucrose gradient. Field flow fractionation can also be used for separating cells according to their size and shape. Adipose tissue cells will separate without centrifugation; they just float. Antibodies raised against any special marker on the cell surface could be explored for selection of that special cell type with various techniques. For example, using metallic iron coupled to antibodies a strong neodymium permanent magnet could be used for selecting a specific cell type. Selective chemical lysis of cell types that are not of interest followed by mild centrifugation will provide the cells containing the enzyme of interest. Even

homogeneous mammalian cells can differ in enzymatic activity if the age or nutritional status of the animals is not identical.

Intact Cells

When a reaction mixture consists only of one type of cell any assay of enzymes on the cell surface will only be straightforward if the cells are not disrupted during determination, because the presence of intracellular components could give rise to false results. For example, if cell-surface adenosine triphosphatase (ATPase) activity is to be measured it is suitable to monitor the product ADP (adenosine diphosphate). However, if the cells are lysed, intracellular ADP will affect the enzyme activity determination.

Subcellular Samples

Depending on the localization of the enzyme of interest, different strategies for the cell lysis have to be considered. A subcellular fractionation could be quite rewarding later in the enzyme purification route. Methods such as sonication, use of a French press, blending or homogenization are useful for the lysis of different types of cells.

For example, a French press is needed for the lysis of bacterial cells with rigid cell walls. The use of a Potter–Elvehjem homogenizer (PTFE (poly(tetra fluoroethylene)) pestle in a glass mortar) for the homogenization of cells with fragile cell membranes is a procedure that will merely break the outer cell membrane, leaving most of the cell organelles intact.

Table 1 Marker enzymes for different subcellular fractions

Subcellular fraction	*Enzyme*
Nuclei	DNA nucleotidyltransferase
Nuclei	Nicotinamide-nucleotide adenyltransferase
Mitochondria	Succinate dehydrogenase
Mitochondria	Cytochrome *c* oxidase
Endoplasmic reticulum	Glucose-6-phosphatase
Lysosomes	Acid phosphatase
Lysosomes	Ribonuclease
Peroxisomes	Catalase
Peroxisomes	Urate oxidase
Plasma membrane	5′-Nucleotidase
Cytosol	Glucose 6-phosphate dehydrogenase
Cytosol	Lactate dehydrogenase
Cytosol	6-Phosphofructokinase

From Price and Stevens (1989), p. 369.

A subcellular fractionation scheme based on centrifugation for mammalian cells is outlined in **Figure 2**.

The enzymatic activity of interest can be followed, together with the activity of marker enzymes, through the subcellular scheme (**Table 1**). The subcellular localization is then predicted and further purification can be performed.

Enzyme Purification Methods

Maintenance of Biological Activity

Soluble proteins, either cytoplasmic or inside organelles, are present in highly concentrated soups

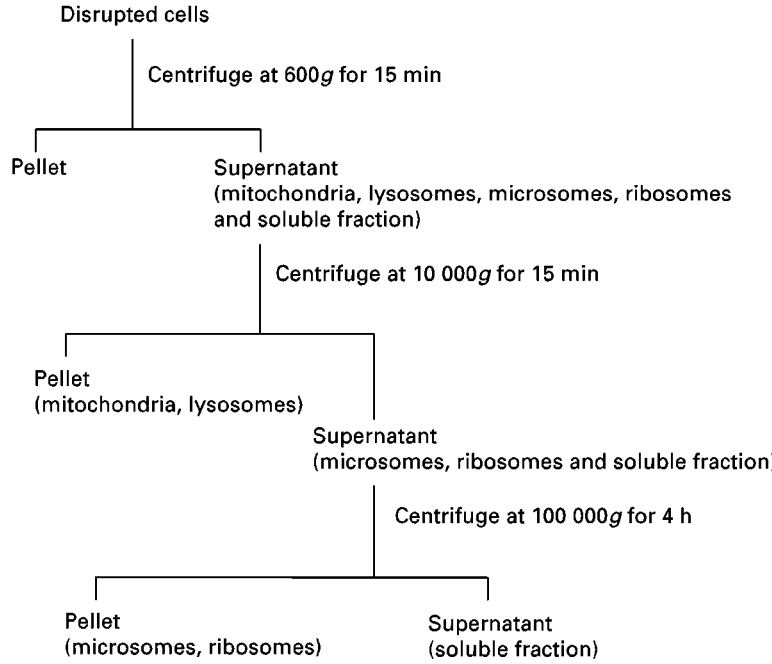

Figure 2 A subcellular fractionation scheme for mammalian cells by differential centrifugation. (From Price and Stevens (1989), p. 368.)

of proteins, with concentrations ranging from 100 mg mL^{-1} to as high as 400 mg mL^{-1} inside the mitochondrial matrix. Oxygen tension is low and different natural reducing compounds such as glutathione are present to maintain a high reducing potential. Other stabilizing agents are also present, such as different substrates and products. When the tissue is disrupted during purification proteins are released from their protective environment and proteolytic enzymes which are held in separate compartments are also released. As a consequence the enzyme of interest has to be protected from oxidation, proteolytic degradation and irreversible unfolding of their tertiary structure. This can be achieved in many different ways and should be tailor-made for each purification scheme and enzyme.

Denaturation during purification can be minimized if precautions are taken according to extremes of pH, temperature and organic solvents. The natural pH inside a cell is normally in the range 6–8. Using buffers within this pH range at appropriate ionic strength should protect against pH denaturation. Reducing the temperature by 15–25°C decreases many degradation processes three- to five–fold. Reducing the temperature also slows down the processes involved in the separation method, for instance in size exclusion chromatography.

Enzymes in dilute preparations may denature due to adsorption onto the wall of the container or onto the chromatography matrix used. Enzymes with quarternary structure will dissociate and the activity of interest will be lost.

Adsorption and denaturation of dilute enzyme samples can be circumvented by using a carrier protein such as bovine serum albumin (BSA), or even better a commercial synthetic carrier protein with a simple and known structure at a concentration as high as 1 mg mL^{-1}. Care must be taken to avoid interaction between the carrier protein and the enzymes of interest. The molecular size of the carrier protein should also differ by at least a factor of two from the enzyme of interest, for easy removal by size exclusion chromatography if the pure enzyme protein is needed, for instance in amino acid sequencing.

Catalytic site inactivation by specific reactions is hard to avoid. Loss of cofactors can be prevented by including them in the purification buffer. Covalent modification of the active site which contains reactive amino acid residues responsible for the catalysis is common. The most troublesome amino acid is cysteine, which is very susceptible to modification. Sulfhydryl residues at the active site may be in the ionized form, which is prone to oxidation and can form disulfide bonds, or may be partially oxidized to the sulfinic acid or irreversibly oxidized to the sulfonic acid.

These reactions can be suppressed by using different additives such as ethylenediaminetetraacetic acid (EDTA), which masks by complexation cations that would otherwise catalyse the formation of disulfide bonds with cysteine residues at the catalytic site. Other commonly used reducing agents are sulfhydryl-containing reagents such as β-mercaptoethanol, 2,3-dimercaptopropanol, thioglycolate, glutathione, cysteine and the dithio-analogues of the reduced C_4 sugars, threitol and erythritol (DTT, DTE). EDTA and other complexing agents often cannot be used for enzymes that have an essential metal ion in the active site.

Proteinases or proteolytic enzymes are contained inside living cells. In mammalian cells they are packed in lysosomes. In microorganisms they are often found between the plasma membrane and the cell wall. During preparation of an enzyme extract by cell homogenization, release of proteolytic enzymes will occur and their activity has to be inhibited. Depending on the wide scale of different proteinases present in the cell homogenate, many types of inhibitors have to be used.

Diisopropyl fluorophosphate (DFP) inhibits serine proteases. Note that DFP is dangerous to handle because it is volatile and attacks human acetylcholinesterase, vital in nerve conduction. Phenylmethylsulfonyl fluoride (PMSF) is a nonvolatile serine protease inhibitor and does not attack acetylcholinesterase. It inhibits some thiolproteases and some carboxypeptidases, but it has to be dissolved in acetone or isopropanol.

Pepstatin, leupeptin and antipain are peptide-based inhibitors that are very potent against acid proteases such as pepsin, cathepsin D and yeast protease A. Normal concentrations of inhibitors used in freshly prepared extracts are 5 mmol L^{-1} EDTA, 1 mmol L^{-1} PMSF, 10 μmol L^{-1} pepstatin, 10 μmol L^{-1} leupeptin and 10 μmol L^{-1} antipain.

Other stabilizing factors during enzyme purification are some nonaqueous hydrophilic molecules. Owing to the high nonaqueous content in cell cytoplasm (10–15% w/v), the water around the protein molecules is not freely mobile and thus stabilizes the protein structure. To mimic this situation in prepared enzyme extracts glycerol is added at 10–50% (w/v). Below 30% the viscosity is not harmful for most methods used during enzyme purification except for ultrafiltration and centrifugation and in some 'salting-out' experiments because hydrogen bonding and hydrophobic forces decrease. Sugar or sugar alcohol solutions such as glucose, fructose, lactose and sorbitol and be used instead of glycerol. The mechanism of protection is similar.

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Separation Methods Based on Size, Shape, Mass, Charge, Hydrophobicity, Solubility and Biological Recognition

The different physicochemical properties of the enzyme that should be utilized during a purification scheme include size, shape, charge, hydrophobicity, solubility and biological recognition. The salient points of various separation methods are listed in **Table 2**.

After each step in a purification scheme, a proper enzyme activity assay should be carried out and the amount of protein determined. If correctly done the specific activity (in units per milligram) (1 U is the amount of enzyme that converts 1 μmol of substrate per min under defined reaction conditions) can be followed through the purification scheme – it should rise and then reach a plateau. Crude extracts should be concentrated by fractional precipitation with ammonium sulfate or poly(ethylene glycol) (PEG) or adsorbed and desorbed from a chromatographic matrix as soon as possible. Precipitated proteins, after dissolution in a small volume, are more stable because they are more concentrated. Centrifugation with field strength from 5000 to 50 000 *g* is widely used for subcellular fractionation and for ammonium sulfate and PEG-precipitated enzymes.

Table 2 Enzyme separation methods

Physicochemical property	*Method*	*Chracteristic*	*Scale*	*Use*	*Enzyme activity recovery*
Size, shape or mass	Centrifugation	Moderate resolution; slow	Large or small	Partial fractionation	Good
	Gel filtration	Moderate resolution; slow	Small	Desalting, size determination, fractionation	Good
	Field flow fractionation	Good resolution; fast	Small	Size determination, fractionation	Good
	Ultrafiltration	Bad resolution; slow/medium	Large or small	Desalting, concentration	Good
	Dialysis	Bad resolution; slow	Large or small	Desalting, concentration	Good
Polarity					
(a) Charge	Ion exchange chromatography	High resolution; fast	Large or small	Fractionation, concentration	Good
	Chromatofocusing	Excellent resolution; fast	Medium	Fractionation, concentration	Poor
	Electrophoresis	High resolution; medium/fast	Small/medium	Fractionation, visualization	Medium/poor
	Isoelectric focusing	Excellent resolution; medium	Small/medium	Fractionation, visualization	Poor
	Capillary electrophoresis	Excellent resolution; fast	Extremely small	Fractionation, size determination possible	Good
(b) Hydrophobic character	Hydrophobic interaction chromatography	Good resolution; fast	Large or small	Fractionation, concentration	Good
	Reversed-phase chromatography	Excellent resolution; fast	Large or small	Fractionation, concentration	Poor
Solubility	Change in pH	Medium resolution; fast	Large or small	Concentration, fractionation	Medium
	Change in ionic strength	Medium resolution; fast	Large or small	Concentration, fractionation	Medium/good
	Decrease in dielectric constant	Medium resolution; fast	Large or small	Concentration, fractionation	Medium
	Two-phase separation	Medium/good resolution; medium	Large or small	Fractionation, concentration	Good
Biological activity	Affinity chromatography	Excellent resolution	Generally small	Fractionation, concentration	Medium/good
Specific binding or structure features	Dye–ligand chromatography	Good resolution; fast	Large or small	Fractionation, concentration	Medium/good
	Immuno-chromatography	Excellent resolution; fast	Generally small	Fractionation	Medium/good
	Covalent chromatography	Medium/good resolution; fast	Medium/small	Fractionation	Medium

Field flow fractionation Field flow fractionation (FFF) is a chromatography-like separation technique which is designed for fractionation of macromolecules, colloids and particles. The principle is simple. A laminar flow of carrier liquid between two walls, separated by *c.* 0.1 mm, creates a parabolic velocity profile. The sample is injected into the carrier stream at the inlet of the channel and exits through the outlet end which is connected to a detector.

Sample retention is achieved when molecules are pushed to the accumulation wall (an ultrafiltration membrane) by an external field force (a cross-flow), so that they obtain different average distances from the wall and are placed at different heights in the parabolic flow profile. The different sample molecules are consequently transported down the channel at different velocities. Thus separation can be achieved.

The size range embraced by FFF is from small proteins (*c.* 10 kDa), up to organelles and cells with a diameter of several micrometers. FFF does not rely on a stationary phase, which makes it very useful for separation of labile enzyme molecules. Separation times are very short (3 – 10 min) and selectivity according to size is better than for gel filtration. Loadability is so far limited to *c.* 200 μg per separation run.

Examples of Enzyme Determination in Physiological Samples

Hormone-Sensitive Lipase from Adipose Tissue

Hormone-sensitive lipase (HSL, EC 3.1.1.3) is an amphiphilic enzyme and the key control of energy substrate flow in mammals. Its activity in adipose tissue determines the rate of hydrolysis of stored triacylglycerols and thereby the production of fatty acids for release as free fatty acids (FFAs) into the circulation.

The following parameters were considered during purification.

1. Development of a suitable assay procedure
2. Selection of the best source from which the molecule could be purified.
3. Solubilization of the desired molecule.
4. Development of a series of isolation and concentration procedures which includes stabilizing the molecule at each stage.

Lipase activity was measured against emulsified [^{3}H]-oleic acid-labelled monooleoylglycerol (a diacylglycerol ether analogue). An enzyme activity of 1 U corresponds to the release of 1 μmol of fatty acids per minute at 37°C. The assay was performed between each purification step. The enzyme source was rat adipose tissue (epididymal fat pads).

A summary of the steps taken in the purification of HSL from rat epididymal adipose tissue is given in **Figure 3**.

Step 1. Fat pads frozen in 0.25 mol L^{-1} sucrose, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} DTE and 10 μmol L^{-1} of leupeptin and antipain in liquid nitrogen were homogenized (Potter–Elvehjem homogenizer) in 30% (w/v) 0.25 mol L^{-1} sucrose and partially delipidated by removing the fat floating after centrifugation at 5000 *g* for 10 min at 4°C.

Step 2. The supernatant was further delipidated and separated from the pelleted material by centrifuging at 100 000 *g* for 45 min (referred to as the '100 000 *g* supernatant').

Step 3. The pH was lowered to 5.2 with acetic acid. HSL was precipitated over 30 min on ice and the pellet collected after 30 min of 10 000 *g* centrifugation. The pellet was resuspended in 20 mmol L^{-1} Tris-HCl (2-amino-2-hydroxymethylpropane–1,3-diol hydrochloride), pH 7.0, containing sucrose as before (this fraction is referred to as 'the pH 5.2 ppt fraction'). This fraction contains practically all the HSL and about 25% of the contaminating proteins. The preparation is stable for several months at − 80°C.

Step 4. Further solubilization of the pH 5.2 ppt fraction was by sonication at 10°C in the nonionic detergent $C_{13}E_{12}$ (heterogeneous alkyl polyoxyethylene glycol type). The solubilized HSL was fractionated by gradient sievorptive chromatography on quaternary aminoethyl (QAE)-Sephadex, for a separation time of 12 h at 10°C. 70% of the recovered enzyme was pooled and concentrated 15-fold by ultrafiltration and referred to as the QAE-Sephadex fraction.

Step 5. This fraction was dialysed and concentrated three-fold further against 20 mmol L^{-1} Tris–acetic acid, pH 7.50, containing 20% (w/v) glycerol, 15% PEG, 1 mmol L^{-1} DTE, 0.2% $C_{13}E_{12}$ and 10 μmol L^{-1} leupeptin for 8 h at 4°C, immediately followed by chromatography on a Mono Q column (polymer-based strong anion exchanger for liquid chromatography from Pharmacia) as described in detail in **Figure 4**. The enzyme peak fractions collected were immediately brought to pH 7.0 by addition of a potassium phosphate buffer to give a final concentration of 30 mmol L^{-1} and the fractions were stored at −80°C in 50% (w/v) glycerol. This enzyme preparation is referred to as the 'Mono Q enzyme'.

Step 6. The last step of the purification scheme consisted of Mono S chromatography (polymer-based strong cation exchanger for liquid chromatography from pharmacia). Before chromatography the Mono Q enzyme was dialysed and concentrated three-fold for 3 h against 10 mmol L^{-1} potassium

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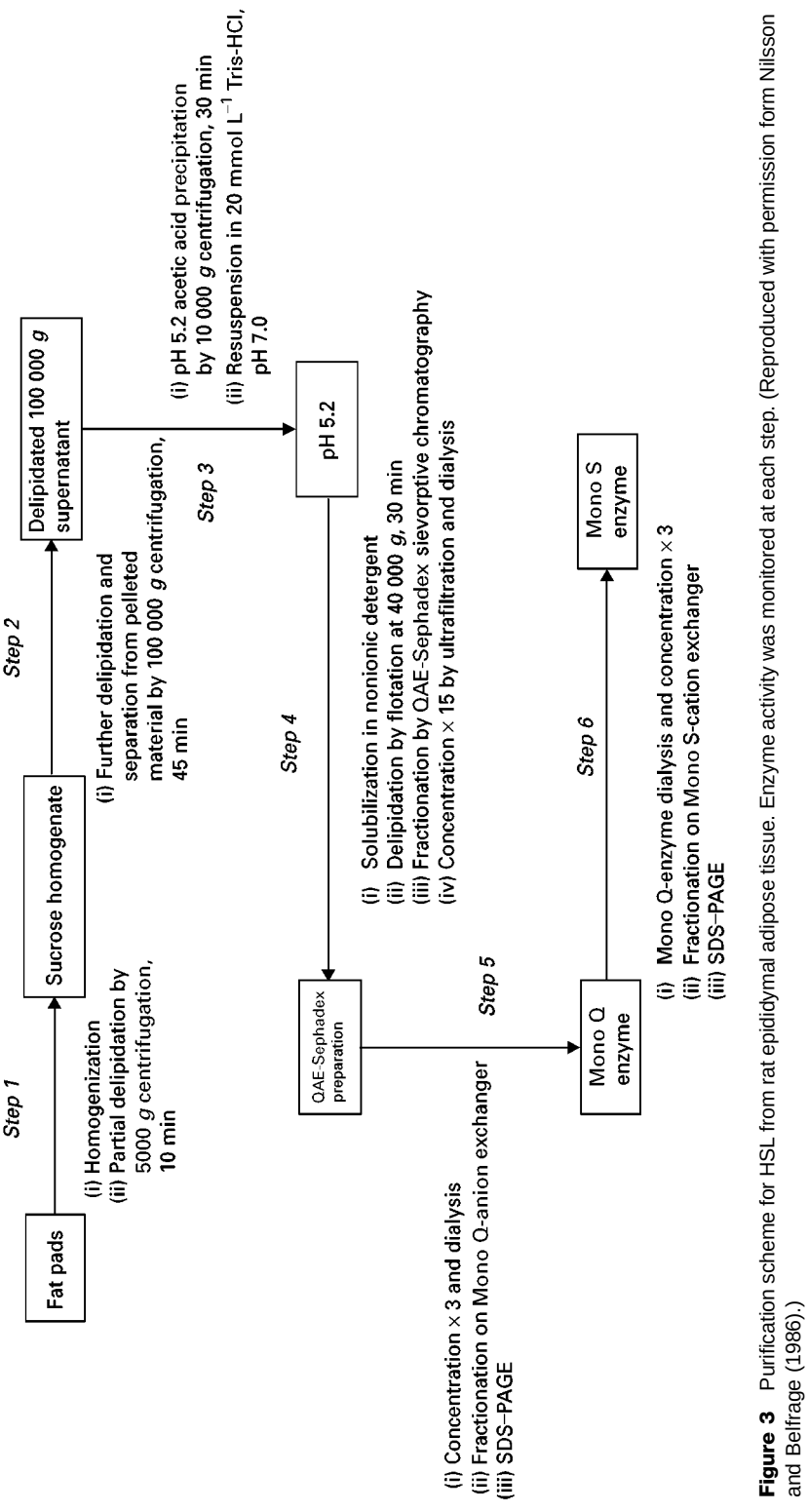
Step 1
Fat pads
(i) Homogenization
(ii) Partial delipidation by 5000 g centrifugation, 10 min
Sucrose homogenate
Step 2
(i) Further delipidation and separation from pelleted material by 100 000 g centrifugation, 45 min
Delipidated 100 000 g supernatant
Step 3
(i) pH 5.2 acetic acid precipitation by 10 000 g centrifugation, 30 min
(ii) Resuspension in 20 mmol L−1 Tris-HCl, pH 7.0
pH 5.2
Step 4
(i) Solubilization in nonionic detergent
(ii) Delipidation by flotation at 40 000 g, 30 min
(iii) Fractionation by QAE-Sephadex sievorptive chromatography
(iv) Concentration × 15 by ultrafiltration and dialysis
QAE-Sephadex preparation
Step 5
(i) Concentration × 3 and dialysis
(ii) Fractionation on Mono Q-anion exchanger
(iii) SDS–PAGE
Mono Q enzyme
Step 6
(i) Mono Q-enzyme dialysis and concentration × 3
(ii) Fractionation on Mono S-cation exchanger
(iii) SDS–PAGE
Mono S enzyme

Figure 3 Purification scheme for HSL from rat epididymal adipose tissue. Enzyme activity was monitored at each step. (Reproduced with permission form Nilsson and Belfrage (1986).)

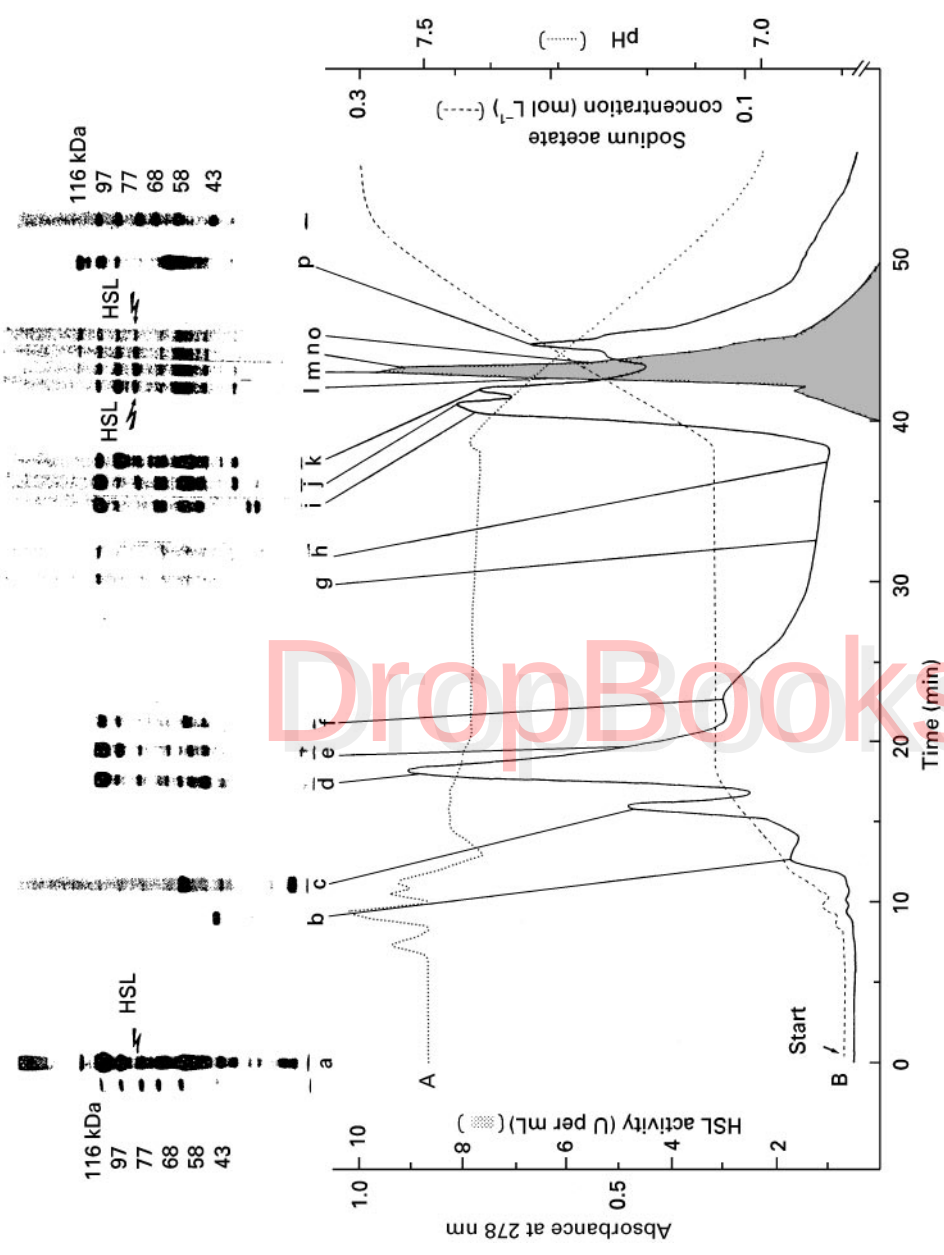

Figure 4 Fractionation of partially purified HSL by high-performance Mono Q anion exchange chromatography. (A) The sample, 7.5 mL of concentrated QAE-Sephadex enzyme (about 17 mg of protein), representing half of the preparation from adipose tissue of 500 rats, was applied to an 8 mL Mono Q column (fitted with a precolumn, flow rate 1.0 mL min^{-1} at a back-pressure of about 1.5 MPa) pre-equilibrated in 50 mmol L^{-1} Tris-acetate, pH 7.5, containing 1 mmol L^{-1} DTE, 20% (w/v) glycerol and 0.2% (w/v) of the nonionic detergent $C_{13}E_{12}$. (B) After adsorption the lipase was eluted (4.0 mL min^{-1} at a back-pressure of about 4.2 MPa) by the increasing salt and decreasing pH gradient obtained by addition of 0.3 mol L^{-1} sodium acetate, pH 7.0, as indicated by the conductivity and pH profile in the figure. HSL activity (shaded area) was measured towards an emulsified lipid substrate. One unit (U) of enzyme activity corresponds to 1 μmol of fatty acid released per minute at 37°C. The protein composition of the QAE-Sephadex enzyme sample applied to the column (lane a), and the indicated column fractions (lanes b–o), analysed by SDS-PAGE and Coomassie blue staining. Lanes to the extreme left and right are reference proteins; values are in kiloDaltons. Arrow labelled HSL signifies the HSL M_r = 84 000 subunit. Enzyme peak fractions corresponding to 70% of the total enzyme eluted were pooled for the next step. (Reproduced with permission from Nilsson and Belfrage (1986).)

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Table 3 Purification of HSL from rat adipose tissue[a]

Purification step	*Volume (mL)*	*Protein (mg)*	*Enzyme activity (μmol fatty acids per min)*	*Specific activity (μmol fatty acids per min per mg protein)*	*Purification (-fold)*	*Yield (%)*
100 000 *g* supernatant	790	4900	314	0.06	1	100
pH 5.2 ppt	50	1204	289	0.24	4	92
QAE-Sephadex	46	33	112	3.40	57	36
Mon Q LC[b]	16	2.1	58	27	450	18
Mono S LC[b]	8	0.2	34	154	2567	11

[a]Enzyme was purified from about 600 *g* of epididymal fat pads from 500 rats. Enzyme activity was measured with monoacylalkylglycerol substrate at 37°C.
[b]Combined enzyme from two identical chromatographic treatments of half the initial batch.
Reproduced with permission from Nilsson and Belfrage (1986).

phosphate buffer, pH 7.0, followed by 3 h against the same buffer, pH 6.5, both containing the same concentration of glycerol, PEG, $C_{13}E_{12}$, DTE and leupeptin as used for the Mono Q chromatography. The enzyme peak fractions (70% of the enzyme activity recovered) were brought to pH 7.0 and glycerol was added to 50% (w/v). The results of the purification are illustrated in **Table 3**.

The obstacles encountered in the determination of enzymes in biological samples are well illustrated in this purification scheme for the enzyme HSL, which has been notoriously recalcitrant to purification because of its low tissue abundance, amphiphilicity and general lability. What are the necessary precautions that have to be fulfilled during the process? After every solubilization, fractionation or concentration step, the biological activity has to be estimated to detect any inhibition, destruction or loss of the enzyme of interest. To obtain optimum enzyme activity, certain precautions have to be taken in between every step as discussed previously, by adding reducing agents or stabilizers, lowering the temperature or speeding up the separations where possible. To elucidate the effectiveness of different fractionation steps used, protein purity must also be examined with a nonchromatographic method such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) or capillary electrophoresis. Another, not easy, problem of importance is the identification of the protein band on SDS-PAGE that corresponds to the enzymatic activity.

In the case of HSL, it was possible to carry out the identification in rather crude preparations because the enzyme was activated through covalent modification, i.e. phosphorylation. Then it was possible to 'tag' HSL with ^{32}P and identify the SDS-PAGE band by autoradiography. After the Mono Q purification step there was only one phosphorylated band.

A single band on SDS-PAGE with Coomassie blue, or even silver staining together with maximum enzyme activity, are not conclusive evidence for identification.

Plasminogen Activator in Gingival Crevicular Fluid

There is a correlation between plasminogen activator (PA) concentration in gingival crevicular fluid (GCF), which is an extracelluar exudate occurring in the gingival crevice, and gingival inflammation. The concentration of plasminogen activator inhibitor (PAI) in GCF also plays an important role. The fibrinolytic system is activated by PAs, which are serine proteases that catalyse the conversion of the inactive proenzyme plasminogen to the active enzyme plasmin which then activates collagenase and thereby participates in the tissue destruction seen at inflammatory lesions.

Sampling of GCF was performed by placing small discs (Millipore GWVP-filter 0.22 μm, calibrated in size to absorb a determined volume) in the gingival crevice.

PA determination is performed by two different methods.

1. Enzyme-linked immunosorbent assay (ELISA), where the amount of protein is determined by placing the small discs in the wells of the microtitre plates used for ELISA (further details of ELISA are given elsewhere). This can be done providing monoclonal or polyclonal antibodies that have been raised against the enzyme, which in turn demands a relatively pure enzyme for immunization.
2. Gel lysis can be used for the determination of enzymatic activity of PA. The filter discs are placed on plasminogen-rich fibrin plates and incubated for 18 h. PA activity can then be derived from the size of the gel lysis.

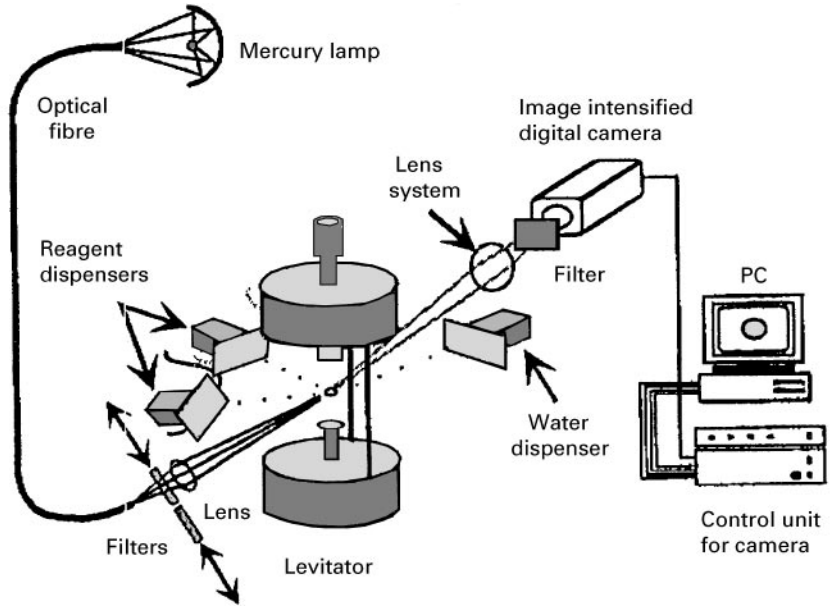

Figure 5 Instrumental set-up for levitation single cell analysis.

Single Cell Analysis

The key step in future enzyme determination is miniaturization, combined with highly selective separation and detection methods. Separation and determination should be done simultaneously. The goal is to be able to study the molecular processes of life at the level of a single cell and its subcellular compartments, if possible without destroying the cell's integrity, and analysing for a specific enzyme. Capillary liquid chromatography and capillary electrophoresis are good candidates for single-cell analysis. In fact, small molecules such as catecholamines, dipeptides and proteins have already been determined in single viable cells. This can be achieved either by sucking a single cell into the capillary and causing cell lysis by use of a high ionic strength buffer, or penetrating the outer cell membrane with an ultrathin capillary (2 μm i.d.) and sucking in some of the cell contents.

By introducing a substrate for a specific enzyme during the separation, it is possible to follow its activity directly after cell lysis by studying the decrease of the substrate and increase of the product concentrations. In some cases the enzyme itself can be monitored during the separation if the amount is high enough. Detection techniques sensitive enough to detect substrates, products and enzymes at the concentration levels derived from a single cell experiment are laser-induced fluorescence or amperometric detection.

Single cell analysis can also be achieved using other miniaturized analysis systems. A very suitable method for studying living cells and biochemical reactions is acoustically levitated microdroplets. Cell studies have for example been performed on freshly prepared, intact and living primary adipocytes. The instrumental set-up is shown in **Figure 5**. A single adipose cell in a 500 nL droplet is acoustically levitated. Stimulation of adipocytes with β-adrenergic agonists results in activation of adenylate cyclase, production of cAMP and activation of cAMP dependent protein kinase (PKA). PKA phosphorylates HSL, leading to activation of enzyme activity and increased lipolysis, resulting in FFA release and a consequent pH decrease in the surrounding buffer droplet. Addition of insulin antagonizes this effect and hence also the decrease in pH. The change in pH, i.e. the cell response in the droplet, is followed by a pH-dependent fluorophore continuously monitored by fluorescence imaging detection. Additions to the levitated droplet are achieved using continuous flow-through droplet dispensers. To counteract droplet evaporation, which affects the fluorescence intensities, a dispenser is used to continually add water, thus keeping the droplet volume constant. An image analysis computer program is employed to calculate droplet fluorescence intensities during experiments. The method is particularly useful for studying of dynamic events in natural cellular environments at the single cell level, e.g. for the screening of new drug candidates or for studying side effects and reactions between cells.

Subcellular fractionation at the single-cell level using a two-phase levitated droplet system is under development at the author's laboratory.

See also: **I/Affinity Separation. II/Affinity Separation:** Theory and Development of Affinity Chromatography.

DropBooks

Centrifugation: Analytical Centrifugation. **Chromatography:** Protein Separation. **III/Enzymes:** Liquid Chromatography. **Proteins:** Capillary Electrophoresis; Centrifugation; Crystallization; Electrophoresis; Glycoproteins: Liquid Chromatography; High-Speed Countercurrent Chromatography; Ion Exchange; Metalloproteins: Chromatography. **Appendix 1/Essential Guides for Isolation/Purification of Enzymes and Proteins.**

Further Reading

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Liquid Chromatography

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Introduction

Enzymes find applications in food, pharmaceutical and biochemical industries. They are found in combination with other macromolecules or various small molecules. Their uses require identification and purification of the enzymes. The nature, quality, and quantity of the desired enzyme are determined by its intended use. For example, the food industry needs enzymes in large quantities and the pharmaceutical industry requires ultra pure enzymes. High-performance liquid chromatography (HPLC) is widely used for the separation or purification of enzymes on a preparative or analytical scale. It is also used for the analysis of the enzymatic activity.

Properties of Proteins and Practical Implications

All enzymes are proteins. All proteins are macromolecules with molecular weights ranging from hundreds to several thousands. The sequence of amino acid in a protein is specific and this gives each protein unique properties. A protein with just amino acids as a building block is a simple protein; those that contain additional units, such as a nucleic acid, a lipid or a metal etc., are called conjugated proteins.

There are 20 naturally occurring amino acids found in proteins that vary in structure; thus, it is the amino acid sequence and composition that determine the properties of enzymes. Two distinct properties are the size and polarity of the protein, which are important factors for understanding their separation. The size of a protein depends upon the number of amino acid units in the protein, whereas the polarity depends on the hydrophilic and hydrophobic units present.

A protein molecule contains one of the three groups: uncharged polar, potentially positively charged (basic side chain) or a potentially negatively charged (acidic side chain). These side chains are normally ionizable and this leads to proteins having characteristic isoelectric points. Since there are other issues governing the protein molecule, such as size, shape and nature of the solution (pH), the overall net charge and polarity depends upon the combination of these factors. In general, the chromatographic processes associated with the properties of enzymes can be summed up as indicated in **Table 1**.

High-Performance Liquid Chromatographic Techniques

Size-Exclusion Chromatography

Size-exclusive chromatography (SEC) is primarily used as a first step in purification when molecules

Table 1 Chromatographic processes associated with enzyme properties

Enzyme property	*Chromatographic method*
Net charge	Ion-exchange chromatogrphy
Size	Size-exclusion chromatography
Substrate affinity and conformation	Affinity chromatography
Polarity	Reversed-phase chromatography and hydrophobic-interaction chromatography